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TITLE: Evaluation of Radiolabeled Tumor Vessel Targeting Peptides as Novel Agents for the Staging and Therapy of Human Prostate Cancer

PRINCIPAL INVESTIGATOR: Bruce R. Line, M.D.
Antonio Passaniti, Ph.D.
Carol Lambert, Ph.D.
Ron Mease, Ph.D.

CONTRACTING ORGANIZATION: University of Maryland, Baltimore
Baltimore, MD 21201

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6. AUTHOR(S)
Bruce R. Line, M.D.
Antonio Passaniti, Ph.D.
Carol Lambert, Ph.D.
Ron Mease, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Maryland, Baltimore
Baltimore, MD 21201

E-Mail: bline@umm.edu

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13. ABSTRACT *(Maximum 200 Words)*

The purpose of this study is to develop a new imaging agent to detect prostate cancer. This will be accomplished by using radiolabeled peptides that bind specifically to receptors on tumor blood vessels. The approach will produce a picture of the tumor for determining the spread of prostate cancer or the agent can be modified to help destroy the tumor.

It is known that the blood vessels that nourish prostate cancers have unique receptors on their surface. Our research group has the expertise to make these peptides and conjugate different radioactive molecules to them. We also have the expertise to grow human prostate cancers in immune deficient mice and evaluate the uptake of radioactive molecules in these tumors by developing through a special camera an image of where the radioactivity is in the tumor-bearing mouse. This collective expertise enables us to test the hypothesis that radiolabeled tumor vessel targeting peptides (TVTP) will selectively bind to tumors and produce a scintigraphic image which will show where the tumor is in the body. Also, by changing the strength of the radioactive molecule on the TVTP, this method will selectively deliver a destructive dose of radioactivity to the tumor.

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Table of Contents

Annual Report 2002

Evaluation of Radiolabeled Tumor Vessel Targeting Peptides as Novel Agents for the Staging and Therapy of Human Prostate Cancer

Background Information:

This annual report covers work performed over the interval from funding restoration (1/29/02) to the date of the required annual report 02/02/02. The initial funding was to cover work from 1/1/00 to 2/2/03, but the principle investigator transferred to the University of Maryland as of 8/1/00 and applied for the transfer of funding to re-establish the grant at the University of Maryland. A report of the work performed at the Albany Medical Center has been submitted to the funding agency. The grant was rewritten for the University of Maryland and the impact statement was submitted (7/27/01).

Although the funding for this project has just been restored, we have carried out preparatory work during the unfunded interval. A good deal of this effort has been in the area of infrastructure redevelopment. We have re-established the chemistry and imaging laboratories in new space as is illustrated in figure 1. This report covers the unfunded preparation and the period of funding (1/29/02-2/2/02).

Introduction:

The purpose of this study is to develop radiolabeled peptides that bind specifically to receptors on tumor blood vessels and use these peptides for radiological detection and treatment of human prostate cancers.

Recent studies have reported on the synthesis of RGD-containing peptides which bind to αV integrins, and which are found in high concentration in the tumor vasculature.[1-4] αV integrins are an ideal target for both diagnostic imaging and directed therapy. They appear early in tumor growth, they are highly selective for the neovascular tissue of tumor, and they are critical to tumor growth and progression. The availability of highly specific targeting peptides that preferentially localize at sites of tumor angiogenesis[1] makes these an excellent choice for delivery of imaging and therapeutic modalities.

Our hypothesis is that radiolabeled tumor vessel targeting peptides will selectively bind to the blood vessels feeding human prostate cancers and provide a novel means for detecting sites of human prostate cancer. To test our hypothesis we will complete the following specific tasks.

1. Synthesize and evaluate tumor vessel targeting peptides (TVTP). Our lead peptide will be ACDCRGDCFCG, since previous reports suggest that this will have a high probability of binding to integrin receptors on tumor vasculature.[1,2] Peptides will be synthesized using FMOC protection on a PerSeptive Pioneer automated peptide synthesizer, purified by reverse phase HPLC and cyclized at high dilution by $K_3[Fe(CN)_6]$ oxidation. A control peptide, ACDCRGECFCG, will be produced in a similar manner. NMR, amino acid analysis and

size exclusion HPLC will verify the structure of the molecules. Radiolabeled molecules will be prepared via MAG3 chelator derivatization for gamma-emittingTc-99m. In vitro biological activity of peptides will be verified using standard human vascular endothelial cell adhesion assays both before and after radiolabeling of peptide.

2. Evaluate tumor imaging with the most promising candidate peptides developed in Task 1 using human prostate cancer xenografts growing in SCID mice. Tracer clearance kinetics, biodistribution and organ residence times of radiolabeled complexes will be evaluated and compared to the control peptide. Studies will assess tumor to background ratio and the percent-injected dose per gram of tumor tissue at 0-1 and 24 hours after tracer administration. One human breast cancer known to localize these peptides (positive control) and four different human prostate cancers will be used in this part of the study.

3. Evaluate therapeutic potential of beta-emission (Rhenium-188) radiotherapy of prostate cancer using TVTP. Using the prostate tumor cell line showing the best TVTP uptake, dosimetry studies will be carried out to evaluate the tumor radiotherapy dose potentially obtainable with Rhenium beta-emission radiotherapy. This data will be obtained from tissue residence constants using Tc-99m radiolabeled peptide biodistribution and necropsy data over 24 hours, since chelation and biodistribution characteristics of Tc-99m and Re-188 are similar.

Body:

The body of this brief report is organized by task as listed above in bold typeface. The work carried out to date is described in normal face type.

Task 1: Months 1-12: Develop and evaluate candidate tumor vessel targeting molecules: RGD-4c and NGR peptides

a) Synthesis and cyclization of peptides. Radio-ligand chemistry. Studies of peptide structure including NMR, amino acid analysis, and HPLC analysis.

In the initial period of grant funding (in Albany) we were able to synthesize the RGD4C (ACDCRGDCFCG), the NGR (CNGRCVSGSAGRC) and the RGE4C (ACDCRGECFCG) peptides with >80% purity. Following the procedures utilized by Ruoslahti et al., we have cyclized the RGD4C peptide forming disulfide bonds via air oxidation at high dilution. The RGD4C and RGE4C compounds were re-analyzed at the University of Maryland. Because the HPLC of the RGE4C showed multiple significant peaks, suggesting degradation, the RGE4C was re-synthesized and air oxidized at the University of Maryland's Biopolymer Core Facility. The NGR (CNGRCVSGSAGRC) has been obtained from SIGMA-GENOSYS and has a purity of >80%.

Radio-ligand chemistry.

Dr. Mease, a co-investigator chemist, developed an improved procedure for the non-aqueous synthesis of S-acetyl NHS MAG3, the radioligand we are using for binding 99mTc to our candidate peptides. He coupled the NHS ester of S-acetyl MAG3 to the RGD peptide. He also developed an HPLC procedure for monitoring the coupling reaction and for purification of the

coupled peptide. The procedure is carried out using a Macrosphere RP300 C18 7 micron column and a gradient of 0.05% TFA/H20 and 0.05% TFA/acetonitrile. We have prepared a large batch of the RGD-MAG3 by an in-situ process just prior to conjugation to the peptide. We have used this procedure to stockpile four grams of ligand.

Peptides are purified using reverse phase size-exclusion chromatography. Identity and structure of the molecules have been confirmed using mass spectroscopy and HPLC analysis.

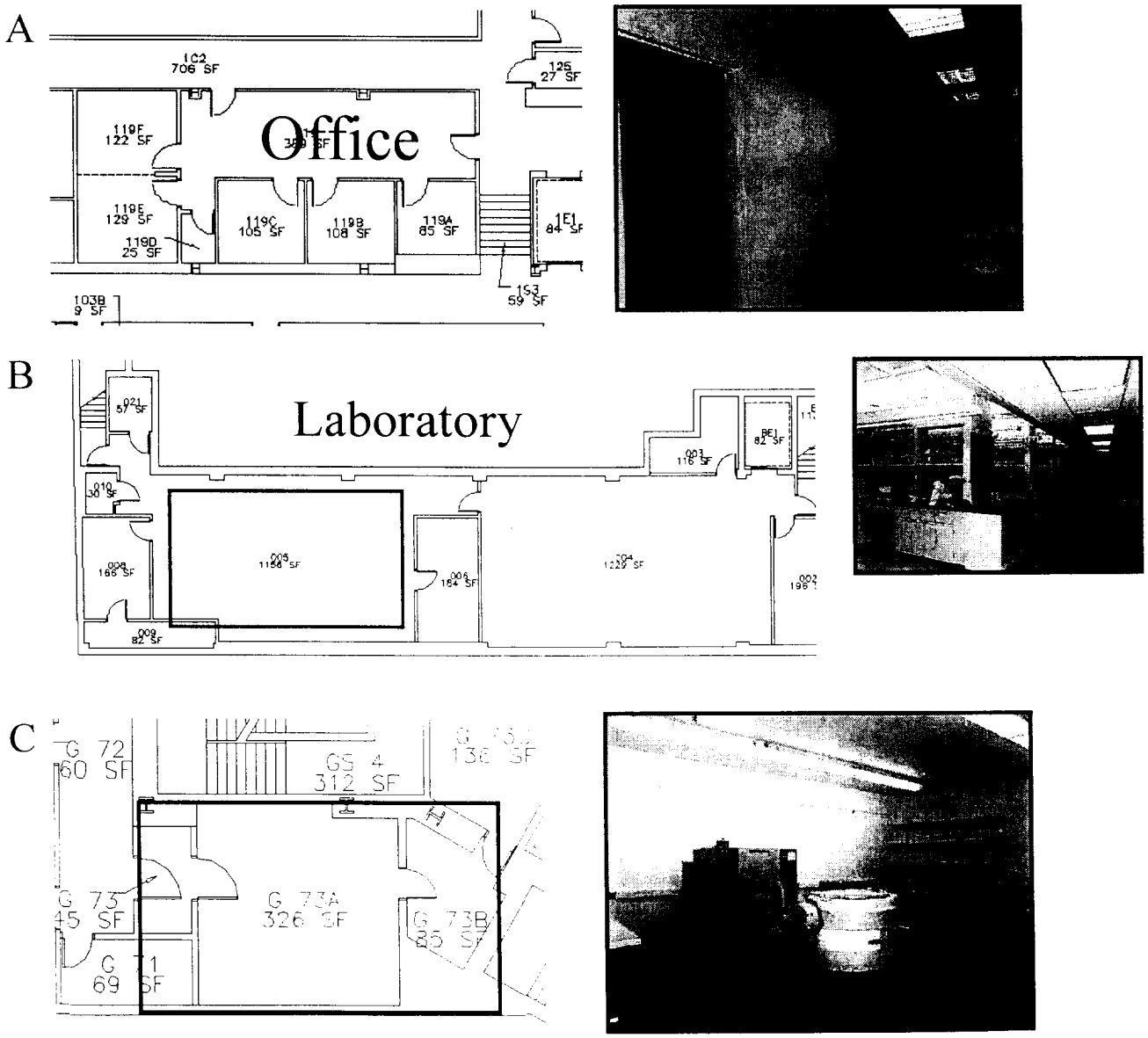

Figure 1. Office, biochemistry laboratory and animal imaging space dedicated for use by the project. Panel A shows the office space and Panel B shows approximately 3500 sq. ft. laboratory space. The 1156 sq ft segment in outlined in red is dedicated to the cell culture and radiolabeling work for this project. Panel C shows the 400 sq.ft. imaging facility (red outline) dedicated to animal research in the Medical School Teaching Facility building.

b) In-vitro studies of the radiolabeled peptides including radiolabeling efficiency, labeling stability via challenge studies at 37°C, bioreactivity measured by cell adhesion assay, HPLC biodegradation studies in plasma at 37°C over 24 hours, and serum protein binding.

In-vitro studies of the radiolabeled peptides including radiolabeling efficiency:

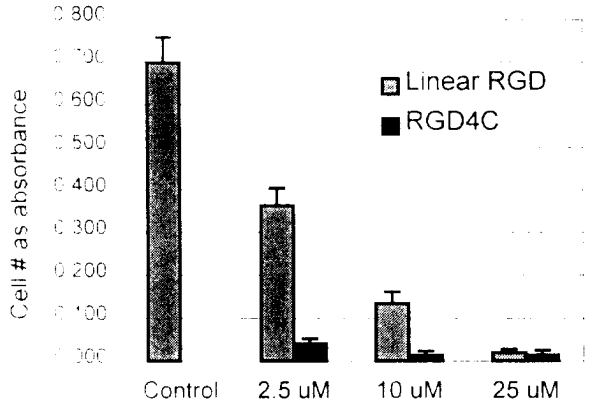

Figure 2. Linear RGD and cyclized RGD4C show concentration dependent inhibition of endothelial cell binding to fibrinogen plated wells. Binding of bovine endothelial cells (expressing the $\alpha v\beta 3$ integrin) is strongly inhibited by RGD4C which demonstrates higher affinity than the linear RGD peptide.

We have collected preliminary information regarding in-vitro stability of the peptides given that storage of peptides, even in a lyophilized form, can affect their bio-efficacy. The RGD4C peptide has been repeatedly tested by mass spectroscopy over a 12 month period without evidence of dimerization. The doubly cyclized structure of RGD4C should impart significant stability. The in vivo stability of the Tc-99m MAG3 has been extensively studied by our collaborator Dr. Hnatowich and has been shown to have no serious degradation or interference with serum components. The tin reduction required to prepare the technetium for chelation to radiolabel the conjugates does not appear to affect the disulfides within the RGD4C. The evidence for this is drawn from the observation that free sulfhydryls exposed during a strong reduction are sufficient to bind technetium, and that unconjugated RGD4C was unable to bind technetium after exposure to tin reduction. Based on preliminary work, we expect our ^{99m}Tc radiolabeled peptide conjugates to have better than 90% labeling efficiency and better than 90% binding stability at 3 and 6 hours post labeling. Prior to *in vivo* studies, radiolabeling efficiency will be checked with thin layer chromatography

Bioreactivity measured by cell adhesion assay

Affinity of the peptides for their receptors is assessed via cellular adhesion assay. Thus far, the RGD-4C (ACDRGDCFCG) has been assessed using a simple plate assay. A 96 well plate is coated with fibrinogen and left to incubate for 1 hour at 37°C. The plate is then blocked with bovine serum albumin for another hour. The vascular endothelial cells and various concentrations of peptide (1ug/ml to 500ug/ml) are then added to the plate for 30 minutes and washed. The cells are fixed with 37% formaldehyde and stained with crystal violet. Finally, citric acid is added at 100ul/well and the plate is read at 540nm on an ELISA plate reader. A quantitative relationship between the amount of peptide and the adherence of the cells was shown to exist. In studies completed to date, adherence of cells was inhibited at a peptide concentration as low as 2.5 uM. (Figure 2) Cell adhesion assays involving the other peptides are underway.

Initial studies were performed in the laboratory of Dr. Passiniti, our coinvestigator. These procedures have been transferred to our new laboratory space (figure 1) where there is a cell incubator and laminar flow hood available for this work.

The rate of degradation and the viability of the chelated radiolabeled peptide will be assessed prior to in vivo analysis. The peptides will be incubated at 37°C with serum over a 24-hour period. Dr. Hnatowich routinely tests peptide radiolabel stability by size-exclusion HPLC analysis. Retention time shifts to higher molecular weight with increased exposure time reveals aggregation with serum components. Retention times indicative of lower molecular weight reveal peptide breakdown products. Although no work has begun at this time, the gamma counting facilities for this portion of the work are available in our laboratory space.

Task 2: Months 13-24: Evaluate tumor angiogenesis imaging using radiolabeled peptides.

a) Carry out biodistribution and tumor uptake studies of the radiolabeled peptides in SCID mouse xenograft models of four human prostate cancer cell lines and one control cell line (MDA-MB-435). Evaluate blood clearance kinetics, biodistribution and organ residence times of radiolabeled complexes. Compare tumor target to background ratio and %ID/gram obtained from imaging at 0-1 hour and at 24 hours for prostate and control tumor xenograft.

Biodistribution and tumor uptake studies of the radiolabeled peptides in SCID mouse xenograft model.

We have successfully grown human prostate cancers in immune deficient mice. Which will allow us to evaluate the uptake of radioactive molecules in these tumors. This expertise also enables us to test the hypothesis that radiolabeled tumor vessel targeting peptides (Tc^{99m} labeled NGR, RGD4C, and RGE4C) will selectively bind to tumors.

The tumors that we are studying include human prostate cancers LnCaP (androgen dependent), LuCap 23.2 (androgen dependent), DU 145 (androgen independent), PC-3 (androgen independent) and human breast cancer MDA-MB-435 (control). These cell lines, purchased from NIH and ATCC, are maintained in culture for use in xenograft implantation. The protocol to implant tumor cells is one used by Dr. Passaniti in which ten million cells per cell line are mixed with matrigel in a 1:1 ratio for a total volume of 500ul. This mixture is injected subcutaneously into the mouse in the region of the brachial lymph. Using that amount of cells allowed us to see tumor growth within three week of injection. Thus far at the University of Maryland, we have grown the PC-3 and the DU-145 xenografts in mice. We will evaluate tumor angiogenesis imaging with candidate peptides about three-six weeks after xenograft implantation, upon which the mice will be injected with the radiolabeled peptide and imaged between 0-60 minutes and 22-24 hours. This will take place in our imaging room in the animal research facility.

We have re-established an imaging facility in the MSTF building as illustrated in Figure 1. This 400 sq. ft. space is located within the animal research facility and contains a gamma camera and associated data acquisition computer systems. This device has been used for SCID mouse imaging studies in other projects. Our collaborative group has demonstrated skills in mouse model anesthesia, scintigraphic imaging and necropsy studies.

Evaluate blood clearance kinetics, biodistribution and organ residence times of radiolabeled complexes.

Tracer clearance kinetics, biodistribution and organ residence times of radiolabeled complexes will be compared to In-111 Prostascint. This portion of the project has not been started to date. The research team established by the principal investigator includes a clinical radiopharmacist who is skilled at radiolabeling Prostascint as we use the material in our clinical Nuclear Medicine practice at the University.

Compare tumor target to background ratio and %ID/gram obtained from imaging at 0-1 hour and at 24 hours for prostate and control tumor xenograft.

Initial biodistribution and metabolism studies have been carried out in SCID mouse xenograft models of four human prostate cancers and a human breast cancer (MDA-MB-435) as a positive control. Our studies will assess tumor to background ratio (T/B) and the %ID/gram at 0-1, 24 hours. These studies will assess the relative uptake of the receptor antagonist peptides.

In-vivo biodistribution studies have been performed in a small number of SCID mice bearing xenografts of DU145 human prostate cancer (Figure 3). Tumor implants were placed in the left axillary region and were imaged when the tumor diameters were greater than 0.5 cm in size. The mice were anesthetized, received 5-15 MBq of radiolabeled test tracer, and were imaged from 0-60 minutes and at 18-24 hours. Although high uptake in the liver and kidney region is evident, there is relatively intense localization in tumors as small as ~60 mg in size. These exploratory trials were performed at the Albany Medical College. The work for this phase of the project has been just started at the University of Maryland.

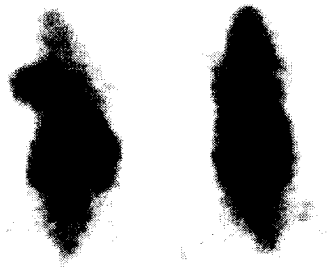

Figure 3. SCID mice bearing a DU145 human prostate cancer in the left axillary region. The tumor diameters are 1 cm (0.5 gm) for mouse on left and 0.5 cm (0.06 gm) for mouse on right. Images obtained at 24 hours post injection with 0.6 mCi of ^{99m}Tc MAG3-RGD4C. Intense uptake is evident in the tumors, liver, kidney and bladder regions.

Given the animal imaging facility, the cell culture and laminar flow hoods in our laboratory facility and our prior experience, we foresee no difficulty in carrying out this portion of the work scope. The necessary software for the image analysis was written by the principal investigator in MS Visual Basic and has been installed on the computers in our lab. Necropsies may be carried out in the animal facility and radioactivity in tissue specimens will be counted on our gamma counter.

b) For each prostate tumor cell line, correlate %ID/gram uptake and target to background (tumor to thigh) ratio to tumor angiogenesis as measured by $\alpha v\beta 3$ integrin prevalence, and parameters from compartment analysis of specific vs. non-specific radiolabeled peptide distribution in normal and xenograft tissues.

Standard descriptive statistics (means and standard deviations) will be used to summarize the %ID/gram and the tumor to background (T/B) results. We will analyze %ID/gram and T/B ratios by repeated measures analysis of variance (ANOVA) with fixed effects of tumor type and tracer. Repeated measures analysis will include an effect of time (for the 60 minute and 24 hour

determinations) as well as interactions (tracer with tumor, tracer with time, tumor with time, and tumor with tracer with time). When justified by statistically significant main or interaction effects, multiple comparisons between tracers within a tumor type will be performed by the Student-Neuman-Keuls multiple range test (using the best time for each tracer). We have not begun work on this portion of the project, but will obtain the services of the biostatistic group at the University of Maryland. We also maintain a good working relationship with the biostatistician at the Albany Medical College.

Task 3: Months 25-36: Evaluate tumor targeting peptides as potential radiotherapeutic agents for human prostate cancer.

a) **Using best tumor and best targeting peptide selected from the candidates evaluated in task 2, perform dosimetry studies to evaluate possibility of beta-emission radiotherapy of tumors via TVTP delivery.**

b) **Use image activity biodistribution and necropsy data over time to determine residence constants using Tc-99m radiolabeled TVTP peptides.**

c) **Assess utility of image count analysis vs. necropsy data to estimate organ dosimetry.**

No work has been performed for this task as yet

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